

## Silk-inspired polymers and proteins

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**Abstract:** The biocompatibility, biodegradability and benign conditions under which natural silk protein fibres (with impressive mechanical properties) are produced represent a biomimetic ideal. This ideal has inspired people in both academia and industry to prepare silk-mimetic polymers and proteins by chemical and/or biotechnological means. This mini-review aims to give an overview of the design principles of such silk-inspired polymers/proteins, their processing into various materials morphologies, their mechanical and biological properties and finally their technical and biomedical applications.

**Keywords:** silkworm silk, spider silk, silk-inspired, biomimetic materials.

**Abbreviations used:** Ala, Alanine; Arg, Arginine; Asp, Aspartic acid; *E. coli*, *Escherichia coli*; Glu, Glutamic acid; Gly, Glycine; HFIP, Hexafluoroisopropanol; MW, molecular weight; PEG, Poly(ethylene glycol); Pro, Proline; RGD, ArgGlyAsp; Ser, Serine; Val, Valine; TM, Trademark; Wt%, percent of weight; Xaa, any amino acid.

## Introduction

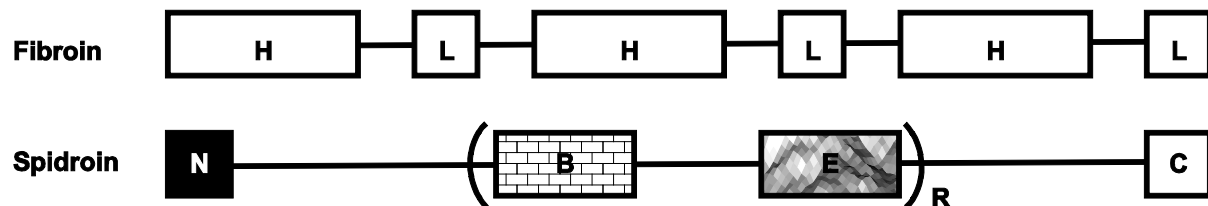
Arthropods have evolved to produce a variety of task-specific silk protein-based fibres. Silkworms produce cocoons from silk protein-based fibres as a means of protection during their metamorphosis into moths, and web-weaving spiders produce a number of different silk protein-based fibres to capture prey (in webs), to protect/preserve their offspring/prey (in cocoons), and as lifelines to escape from predators; and certain silk fibres have mechanical properties superior to Nylon, Kevlar and high-tensile steel. Naturally occurring silkworm and spider silk fibres have been used by humans for millennia for applications as diverse as currency, hunting (bow strings, fishing lines or nets), paper, textiles and wound dressings owing to their mechanical properties and biocompatibility [1-3].

**Table 1. Mechanical properties of natural silks and man-made fibres**

Material	Tensile strength (MPa)	Elongation at break (%)	Toughness (MJ m <sup>-3</sup> )
<i>Bombyx mori</i> cocoon silk	600	18	70
<i>Araneus diadematus</i> lifeline silk	1100	27	160
Nylon	900	18	80
Kevlar 49 <sup>TM</sup>	3600	2.7	50
High-tensile steel	1500	0.8	6

Silk fibres are typically composite materials, composed primarily of silk proteins (to which they owe their mechanical properties) and other associated molecules (such as glycoproteins and lipids). Silkworm fibres are composed of 2 microfilaments embedded in a glycoprotein (sericin) coating. The microfilaments comprise a 6:6:1 complex of 3 different proteins: heavy chain fibroin (which is hydrophobic with a molecular weight (MW) of ca. 350 kDa) that is linked to light chain fibroin (which

is relatively hydrophilic with a MW of ca. 25 kDa) via disulfide bonds, and P25 protein (MW of 30 kDa) that is linked via hydrophobic interactions. A spider's lifeline, in contrast, has a core-shell type structure slightly different to the structure of silkworm silk; the core filament is an inhomogeneously distributed polymer blend of mainly 2 proteins (spidroins) that is coated with glycoproteins and lipids [1, 4].



**Figure 1. Highly simplified schematic representation of the primary structures of silk proteins: in the case of silkworm fibroin, H represents the heavy chain fibroin (containing many repeats of the  $\beta$ -sheet forming GlyAlaGlyAlaGlySer hexapeptide), L represents the light chain fibroin; in the case of spider spidroin, N represents the amino-terminus non-repetitive amino acid sequence, B represents the alanine rich blocks of  $(\text{Ala})_n$  and  $(\text{GlyAla})_n$  flanking them that are known to form  $\beta$ -sheet structures, E represents the blocks of  $(\text{GlyGlyAla})_n$  that form  $3_1$ -helices and/or  $(\text{GlyProGlyXaaXaa})_n$  that form  $\beta$ -turn spirals that impart elasticity/flexibility to the proteins, R represents the number of repetitive blocks of B/E, and C represents the carboxy-terminus non-repetitive amino acid sequence.**

The natural process of production (known as spinning) for silkworms and spiders is highly complex. Silk proteins are stored within the animal as remarkably highly concentrated protein solutions (up to 50 wt %) without the occurrence of undesirable aggregation. When necessary, the protein solution can be transported through a duct in which the protein solution is subjected to chemical and mechanical stimuli (such as ion exchange, extraction of water, acidification and elongational flow) that promote protein assembly into fibres.

The impressive mechanical properties of silkworm and spider silk fibres are due to the fact that they are relatively elastic matrices of protein toughened by anisotropic nanoparticulate inclusions (formed from stacks of  $\beta$ -sheets). In the case of *Bombyx mori* silkworm fibroin the GlyAlaGlyAlaGlySer hexapeptide repeat is known to form  $\beta$ -sheets, whereas the fibroins of *Antheraea pernyi* and *Samia cynthia ricini* silkworms and spidroins of spiders are  $\beta$ -sheet rich due to repetitive stretches of poly(alanine). The toughness of spider silks is greater than that of silkworm silks because the anisotropic  $\beta$ -sheet inclusions in spider silk are smaller and better aligned with the long axis of the due to the post-spin drawing process carried out by spiders.

Mankind has been able to cultivate *Bombyx mori* silkworms in captivity and harvest their silk for thousands of years, but attempts to do the same with spiders have proven unsuccessful due to their cannibalistic nature. The biocompatibility, biodegradability and benign conditions under which high-performance fibres are produced have inspired the preparation of silk-mimetic polymers and proteins.

### **Silk-inspired polymers**

Natural silk fibres can be crudely described as matrices of relatively elastic protein toughened by  $\beta$ -sheet nanoparticles, formed by the controlled self-assembly of silk proteins under specific chemical/mechanical conditions. Therefore, silk fibres are nanoparticle-reinforced composite materials. Studies of the self-assembly of a number of silk protein inspired copolymers (containing blocks of  $\beta$ -sheet forming peptides [GlyAla]<sub>n</sub> or Ala<sub>n</sub>) have been reported in the last decade.  $\beta$ -sheet rich fibres or films have been prepared from: triblock copolymers composed of PEG and either [(AlaGly)<sub>3</sub>-GluGly]<sub>10</sub> or [(AlaGly)<sub>3</sub>-GluGly]<sub>20</sub> [5-7]; multiblock copolymers composed of poly(isoprene) and Ala<sub>5</sub>-spacer-Ala<sub>5</sub> [8]; and brush copolymers prepared from acrylonitrile and silk peptides (derived from the reaction of acryloylchloride with chymotrypsin degraded *Bombyx mori* fibroin) [9-11].

However, the effect of the non-peptidic blocks on the mechanical properties or biodegradability of these materials has not yet been reported.

The Sogah group have reported the most comprehensive studies of silk-inspired polymers.

Multiblock copolymers (with MWs between 27 and 40 kDa) composed of a rigid aromatic spacer (derivatives of phenoxathiin or xanthene) that templated parallel or antiparallel  $\beta$ -sheet formation of 2 pendant GlyAlaGlyAla tetrapeptides, linked to flexible blocks (of short alkane or ethylene glycol chains). Films cast from formic acid solution were  $\beta$ -sheet rich but very brittle [12, 13]. Replacement of the rigid aromatic spacer with a flexible poly(ethylene glycol) (PEG) spacer improved the solubility of the polymers to up to 50 wt% in trifluoroethanol. Films cast from trifluoroethanol solution had markedly improved mechanical properties (tensile modulus ca. 225 MPa, tensile strength ca. 14 MPa, elongation at break ca. 21%) due to the increased flexibility of the PEG matrix [14].

The same group prepared multiblock copolymers (of ca. 20 kDa) composed of PEG and either Ala<sub>4</sub> or Ala<sub>6</sub> peptides. Films cast from 40 wt% HFIP solution of the polymer containing the Ala<sub>4</sub> tetrapeptide had better mechanical properties (tensile modulus ca. 310 MPa, tensile strength ca. 17 MPa, elongation at break ca. 26%) than those of the equivalent polymer containing the GlyAlaGlyAla tetrapeptide. Increasing the length of the poly(alanine) stretch to six amino acids improved the tensile modulus and tensile strength to ca. 490 and 19 MPa respectively, yet decreased the elongation at break to 12%.  $\beta$ -sheet rich fibres with  $\mu$ m scale diameters were prepared by extrusion of 5-15 wt% HFIP solutions into a methanol-acetone coagulant bath (a process known as wet-spinning) the elongation at break of such fibres were similar to natural silks, yet their tensile strengths (ca. 14 MPa) were much poorer than natural silk [15].

The high strength and directionality of hydrogen bonding interactions in organic solvents can be used to direct the self-assembly of molecules into supramolecular polymers. For example, conjugation of alkyl chains to the termini of the GlyAlaGlyAla tetrapeptide, yielded molecules that form supramolecular polymers in organic solvents (such as acetonitrile or toluene). The

supramolecular polymer nanofibrils (of less than 20 nm) hierarchically assembled into bundles (with widths of ca. 100 nm) and subsequently into entangled networks of fibres with lengths of several micrometers, ultimately resulting in gelation of the solvent [16]. Alternatively, conjugation of the GlyAlaGlyAlaGly pentapeptide to either one or both termini of an oligothiophene (4-mer) yielded molecules that form supramolecular polymers in organic solvents (such as dichloromethane) that may have interesting nanoelectronic applications [17, 18].

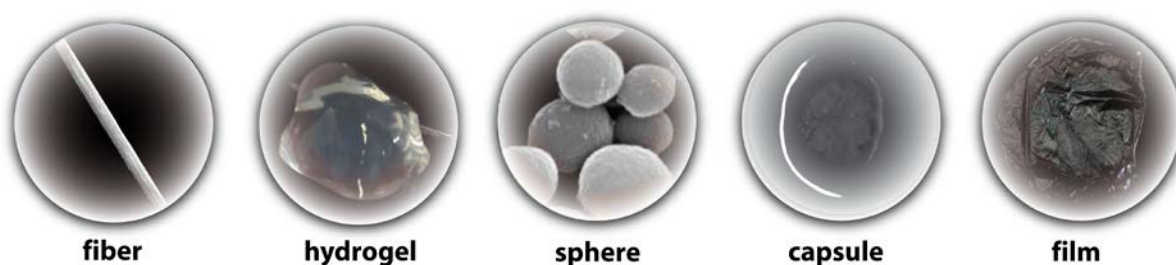
### **Silk-inspired proteins**

Although solid phase synthesis would theoretically allow the preparation of proteins with accurate primary sequences, the high molecular weights of naturally occurring silk proteins (MW > 100 kDa) make this approach impractical. In contrast, fermentation of recombinantly 'engineered' silks is a more practical approach due to its potential for large scale production of proteins with precisely designed primary sequences, potentially allowing production of proteins incorporating entirely new functionality (bioactivity, catalytic properties etc...).

Poly(AlaGly)<sub>64</sub>, poly(AlaGly)<sub>240</sub> and poly([AlaGly]<sub>3-6</sub>GluGly) were produced by high cell density fermentation of recombinant *E. coli* in yields of up to 1 g per liter. Solutions of the proteins in formic acid formed gels upon dilution with water (to a 70% formic acid solution) and washing the gels with methanol yielded a  $\beta$ -sheet rich powder [19-21]. Hydrophobic derivatives of the analogous poly([GlyAla]<sub>3</sub>GlyXaa) (e.g. Xaa = phenylalanine) were insoluble in water even in strongly denaturing conditions, whereas those with polar or charged residues (e.g. Xaa = tyrosine or lysine) could be dissolved in aqueous solutions of denaturants and were found to remain soluble after dialysis against pH buffered water, allowing their subsequent chemical modification. The assembly properties of the glutamic acid derivatives were demonstrated to be pH responsive, aggregating swiftly due to hydrophobic interactions upon protonation of the glutamic acids. Moreover,

adsorption of amphiphilic derivatives ([GlyAla]<sub>3</sub>GlyGlu[GlyAla]<sub>3</sub>GlyLys)<sub>28</sub> to surfaces was shown to render hydrophobic surfaces hydrophilic, and hydrophilic surfaces hydrophobic [19, 22, 23].

While a number of research groups have prepared spider silk-inspired proteins, we are particularly interested in the production of proteins based upon the consensus sequences of the major ampullate silks of *Araneus diadematus* spiders (ADF-3 and ADF-4) that contain  $\beta$ -sheet forming blocks of Ala<sub>6</sub> or Ala<sub>8</sub> and are known as eADF-3 and eADF-4 respectively [24]. Our proteins (of between 46 and 106 kDa) can be produced in high yield by high density fermentation in *E. coli* (or other suitable hosts such as yeast) and purified without the need for chromatographic separation which can be expensive and time consuming [25-27]. Our proteins are soluble in a variety of aqueous and non-aqueous solvents, which facilitates their processing into several different materials morphologies [1] including: fibres [28], films [29-32], foams [33], gels [34], capsules [35, 36] and spheres [37-39]. We have shown that the non-repetitive peptide sequence at the Carboxy-terminus of the proteins encourages their self-assembly [27], and that materials composed of blends of our proteins have improved materials properties [30]. We have shown our materials to be biodegradable [35] and furthermore demonstrated the ability to chemically modify our proteins, allowing us to either reinforce our materials by cross-linking [34], or impart novel functionality via immobilisation of bioactive moieties such as enzymes [32].



**Figure 2. Examples of various materials morphologies preparable with silk-inspired polymers and proteins**



### Triggered assembly of silk-inspired polymers/proteins

Natural silk proteins can be stored at high concentrations without the onset of undesirable aggregation inside the silkworm or spider. When necessary the silkworm or spider exposes the proteins to chemical and mechanical stimuli triggering protein assembly into fibres in a precisely controlled manner [28]. *In vitro*, self-assembly of silk-inspired polymers/proteins due to the formation of  $\beta$ -sheets is typically induced by exposure to a solvent such as an aqueous solution of potassium phosphate known to salt out the protein (as occurs during the natural spinning process) [28, 37, 39] or methanol which dehydrates the  $\beta$ -sheet forming peptides within the polymer/protein [29]. Other stimuli such as stretching (mimicking post-spin draw applied by spiders) [40], storage [41], heat [42] or ultra-violet radiation [41] have also been shown to induce  $\beta$ -sheet assembly.

An alternative to this is self-assembly triggered by exposure to chemical stimuli that subsequently lead to a change of the chemical structure of the polymer/protein. For example as-synthesised silk-inspired triblock copolymers (composed of methyl methacrylate and *tert*-butoxycarbonyl-AlaGlyAlaGly-ethyl methacrylate) had no well-defined secondary structure, as the *tert*-butoxycarbonyl group acted as a steric barrier to  $\beta$ -sheet formation, exposure of the polymers to trifluoroacetic acid led to acid catalysed deprotection of the *tert*-butoxycarbonyl group allowing the formation of  $\beta$ -sheets [5, 43]. Proteins incorporating methionine residues near the  $\beta$ -sheet forming Ala<sub>5</sub> peptides were demonstrated to assemble in solution into nanofibrils due to  $\beta$ -sheet formation. Oxidation of the methionine residues to sulfoxides disrupted the  $\beta$ -sheets yielding fully soluble protein; and reduction of the oxidised residues triggered  $\beta$ -sheet formation and protein-assembly [44-46].

Another alternative is self-assembly triggered by exposure to biological stimuli such as enzymes. This principle was first proven with proteins incorporating phosphorylation sites near the  $\beta$ -sheet forming

Ala<sub>5</sub> peptides. Phosphorylation of the serine residues (with cyclic adenosine monophosphate dependent kinase) yielded highly soluble protein, and subsequent dephosphorylation (with calf intestinal alkaline phosphatase) triggered  $\beta$ -sheet formation and protein-assembly [46-48]. Subsequently, short proteins incorporating the consensus repeats of the major ampullate silks of *Euprostenops australis* spiders (containing long stretches of poly(alanine) [Ala<sub>12</sub>-Ala<sub>15</sub>] that are highly prone to aggregation), the non-repetitive Carboxy-terminus from *Nephila clavipes* spiders (which we have previously shown to play a proactive role in silk protein assembly processes) and thioredoxin (a solubility enhancing fusion protein which prevents assembly of the proteins in solution) were prepared. Proteolytic cleavage of thioredoxin with thrombin triggered the assembly of  $\beta$ -sheet-rich fibres that were hundreds of centimetres in length and had  $\mu$ m scale diameters [49].

### **Silk-based protein chimeras/hybrids**

A variety of silk-based chimeric/hybrid proteins have been produced in the hope of generating proteins incorporating the attractive properties of both proteins, leading to proteins with enhanced solubility, improved cell adhesion or capable of inducing biomineralisation.

Natural *Samia cynthia ricini* silk (whilst biocompatible and biodegradable) is virtually insoluble in both aqueous and non-aqueous solvents (due to repetitive stretches of Ala<sub>12-13</sub>) and therefore difficult to process into materials. Hybrid proteins (MWs between 17-36 kDa) based upon *Samia cynthia ricini* and *Bombyx mori* fibroins were sufficiently soluble to be able to process the resulting protein into fibres [50, 51]. Natural elastins (present in connective tissues) tend to be highly insoluble, rendering processing into materials a challenge. Hybrid proteins (MWs between 45-85 kDa) incorporating silk-like (GlyAlaGlyAlaGlySer) and elastin-like (GlyValGlyValPro) blocks are soluble in a variety of solvents facilitating their processing into fibres, films and gels, that are potentially suitable for various biomedical applications [52].

Hybrid proteins combining  $\beta$ -sheet forming peptide sequences (of either *Bombyx mori* or *Samia cynthia ricini* fibroins) and cell adhesive peptide sequences (such as the ArgGlyAsp (RGD) peptide sequence of fibronectin) were processable into fibres (with either  $\mu\text{m}$  or  $\text{nm}$  scale diameters) or films. *In vitro* cell-adhesion studies demonstrated markedly improved cell adhesion over natural silk proteins with mouse fibroblast [BALB/3T3] cells, human dermal fibroblast [NHDF] cells or African green monkey kidney [VERO] cells [53-56]. Incorporation of the RGD sequence into engineered proteins based upon *Nephila clavipes* spider dragline silk was subsequently shown to improve cell adhesion of mouse osteoblast MC3T3-E1 cells [57].

Hybrid proteins based upon the combination of *Nephila clavipes* spider dragline silk and the R5 peptide (which is derived from the repetitive motif found in silaffin proteins that are involved in silica formation at neutral pH in *in vitro* studies) were processed into fibres or films. Incubation of these materials with a water soluble silicon species led to biomineralisation of their surface [58]. Likewise, chimeric proteins based upon *Nephila clavipes* spider dragline silk and dentin matrix protein 1 (which is involved in the nucleation and oriented crystallisation of hydroxyapatite) could be processed into films. Incubation of the films in simulated body fluid led to the growth of hydroxyapatite crystals on their surface, whereas silk films without the dentin matrix protein 1 domain did not induce biomineralisation [59].

## Conclusions

We are convinced that the production of silk-inspired polymers/proteins aid fundamental research of natural self-assembly processes of silk proteins, and has significant potential for technological and biomedical applications. We envisage that in the future *de novo* designed proteins inspired by silks will have a significant positive impact upon our daily lives.

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